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Dollar Eggs at Fertilization Depends on the Intracellular pH Rise

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The mechanism of the activation of intracellular proteasomes at fertilization was measured in living sand dollar eggs using the membrane-impermeant fluorogenic substrate, succinyl-Phe-Leu-Arg-coumarylamido-4-methanesulfonic acid. When the substrate was microinjected into unfertilized eggs, the initial velocity of hydrolysis of the substrate (V_0) was low. V_0 measured 5 to 10 min after fertilization was five to nine times the prefertilization level and remained high throughout the first cell cycle. Hydrolysis of the substrate was inhibited by *clasto*-lactacystin *β*-lactone, a specific inhibitor of the proteasome. There has been *in vitro* evidence that calcium may be involved in regulation of proteasome activity to either inhibit the increase in peptidase activity associated with PA 28 binding to the 20S proteasome or stimulate activity of the PA 700–proteasome complex. Since both intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) and intracellular pH (pH_i) increase after fertilization, hydrolysis of the proteasome substrate was measured under conditions in which $[Ca^{2+}]_i$ and pH_i were varied independently during activation. When the pH_i of unfertilized eggs was elevated by exposure to 15 mM ammonium chloride in pH 9 seawater, V_0 increased to a level comparable to that measured after fertilization. In contrast, $[Ca^{2+}]_i$ elevation without pH_i change, induced by calcium ionophore in sodium-free seawater, had no effect on V_0 in the unfertilized egg. Moreover, when unfertilized eggs were microinjected with buffers modulating pH_i, V_0 increased in a pH-dependent manner. These results indicate that the pH_i rise at fertilization is the necessary prerequisite for activation of the proteasome, an essential component in the regulation of the cell cycle.

INTRODUCTION

A fully developed egg or oocyte is metabolically quiescent until fertilization initiates the program of development (Monroy, 1965). Egg activation has been extensively studied in the echinoderms, especially in the sea urchin, *Lytechinus pictus.* These studies have shown that activation of metabolism at fertilization, or by parthenogenetic treatment, results from intracellular calcium release (Steinhardt and Epel, 1974; Steinhardt *et al.,* 1974, 1977; Zucker *et al.,* 1978). Since then, with very few exceptions, experimental attention has been focused on how the sperm triggers this initial calcium release. What has been neglected is study of potential targets of the ionic changes at fertilization that lead to activation of metabolism. A promising start has recently been made by Yokosawa and coworkers, who observed an increase in proteasome activity in extracts derived from *Xenopus* oocytes exposed to calcium ionophore, with peak activity for exposures between 2 and 5 min (Aizawa *et al.,* 1996). Their interpretation was that proteasome assembly, and therefore activation of development, was a likely consequence of the calcium transients at fertilization and other points in the cell cycle. Although measurements in living cells have shown that ions, especially free calcium ion, play an important role in cell cycle regulation (Steinhardt, 1982; Poenie *et al.,* 1985; Steinhardt and Alderton, 1988; Wilding *et al.,* 1996; Becchetti and Whitaker, 1997; Groigno and Whitaker, 1998),

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their role in the activation of the proteasome at fertilization has not been studied *in vivo.*

The large size, ease of culture, and optical clarity of many marine invertebrate eggs make them excellent systems for the *in vivo* study of ionic activation of the proteasome at fertilization. Like sea urchin eggs, unfertilized sand dollar eggs are arrested in the G1 phase of the cell cycle. Sand dollar fertilization results in a transient increase in intracellular free Ca^{2+} concentration (Hamaguchi and Hamaguchi, 1990), similar to that in the sea urchin egg (Steinhardt *et al.,* 1977). Studies in sea urchin eggs have shown that the initial calcium release is followed by a sodium-dependent pH rise. (Johnson *et al.,* 1976; Shen and Steinhardt, 1978, 1979), which is essential for the full activation of protein synthesis (Grainger *et al.,* 1979; Winkler *et al.,* 1980). The combined effects of Ca^{2+} and pH_i[2] elevation activate the egg and initiate development (reviewed by Whitaker and Steinhardt, 1982, 1985). In this report we show that in the sand dollar egg calcium release is also followed by a sodium-dependent pH rise. We further investigated whether it is the calcium or pH rise, or both, that is responsible for the activation of the proteasome we observed here *in vivo* at fertilization.

Although much has been learned from *in vitro* and cell-free extract studies of the proteasome (see excellent review by Mykles, 1998), measurement of proteasome activity in living cells in real time should reduce artifacts caused by cellular disruption and isolation from regulatory elements. Recently, a sensitive and continuous assay of proteasome activity in single living cells was developed using the fluorogenic substrate succinyl-Phe-Leu-Arg-coumarylamido-4-methanesulfonic acid (Suc-Phe-Leu-Arg-CAMS) (Chiba *et al.,* 1997). When the substrate is cleaved by the proteasome, a water-soluble fluorescent amine, 7-aminocoumarin-4-methanesulfonic acid (ACMS), is released. Since ACMS is membrane impermeant, the accumulation of ACMS in a single cell can be quantified using a microscope and a photomultiplier.

Using this method we were able to measure proteasome activity *in vivo* during activation of sand dollar eggs. After activation by fertilization, both $[Ca^{2+}]_i$ and pH_i rise, while treatment with calcium ionophore in sodium-free seawater increases intracellular free calcium without elevating intracellular pH. We also studied the effect of artificial increase in pH_i by treatment of unfertilized eggs with ammonia in pH 9 natural seawater or by injection of unfertilized eggs with pH buffers at the more alkaline values found after fertilization.

[2] Abbreviations used: ACMS, 7-aminocoumarin-4-methanesulfonic acid; BCECF, 2′,7′-bis[2-carboxyethyl]-5-[and-6]-carboxyfluorescein; $[Ca^{2+}]_i$, intracellular free calcium ion concentration; pH_i, intracellular pH; Pipes, piperazine-N,N′-bis[2-ethanesulfonic acid]; Suc-Phe-Leu-Arg-CAMS, succinyl-Phe-Leu-Arg-coumarylamido-4-methanesulfonic acid; Zero-Na^+ ASW, zero-sodium artificial seawater.

MATERIALS AND METHODS

Animals

The sand dollar, *Clypeaster japonicus,* was collected off the Pacific coast of Honshu and kept in laboratory aquaria supplied with circulating seawater at 17°C. Animals were used within 2 weeks of collection. Gametes were obtained by intracoelomic injection with 0.5 M KCl or with 50 μl of 50 mM acetylcholine in glass-distilled water. Eggs were collected in seawater, passed through 80-μm mesh to mechanically remove the jelly coat, washed four to five times with natural seawater, and stored at 20°C. Sperm was collected dry, stored at 4°C, and diluted 1:10000 in seawater just before being added to the microscope chamber. Fluorescence was continuously recorded before, during, and after sperm addition. Artificial activation used the calcium ionophore, ionomycin (Sigma, St. Louis, MO). Ionomycin was dissolved in dimethyl sulfoxide at 1 mM to yield a 1000× stock solution.

Solutions for Injection

In Figs. 1 and 3, ACMS (0.25 mM) and bestatin (32 mM) were dissolved in distilled water. Bestatin was used to inhibit exopeptidase hydrolysis of the fluorogenic substrate (Chiba *et al.,* 1997). Suc-Phe-Leu-Arg-CAMS (Chiba *et al.,* 1997), dissolved in dimethyl sulfoxide at the concentration of 25 mM, was diluted with 9 volumes of 100 mM Hepes (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]) buffer, pH 7.0, to make 2.5 mM Suc-Phe-Leu-Arg-CAMS solution for microinjection. The ACMS + bestatin solution was microinjected first, followed by microinjection of the substrate solution. These sequential injections were each 2.5% of the total egg volume. In Tables 1, 2, and 3, the injection solution contained 1.5 mM Suc-Phe-Leu-Arg-CAMS, 3% dimethyl sulfoxide, 0.2 mM Calcium Green, 10-kDa dextran (Molecular Probes, Eugene, OR), 12.5 mM bestatin, 29 mM Hepes, pH 7. Calcium Green dextran was included in the injection solution to monitor injection volume. The volumes injected were about 5% of the total egg volume.

To buffer pH_i (Fig. 3), 300 mM Hepes, 300 mM Pipes (piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]), and 100 mM EGTA (ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid) were dissolved in distilled water and pH was adjusted to 7.1, 7.3, 7.5, 7.7, and 8.0 using KOH. The dextran (10 kDa) conjugate of BCECF (2′,7′-bis[2-carboxyethyl]-5-[and-6]-carboxyfluorescein) (Molecular Probes), 2 mM, was dissolved in 100 mM potassium aspartate and 20 mM Hepes at pH 7.2. The volumes injected were 1% of the total egg volume.

Artificial Seawater

NH_4Cl was dissolved in seawater at 15 mM just before use and pH was adjusted to 9.0 using 1 M NaOH. Sodium-free artificial seawater containing 480 mM choline chloride, 55 mM $MgCl_2$, 10 mM $CaCl_2$, 5 mM KCl, 2.5 mM $KHCO_3$, pH adjusted with 1 M KOH, was used to inhibit Na^+/H^+ exchange. Unfertilized eggs were changed to nominally sodium-free artificial seawater by allowing the eggs to sediment at 1× gravity through six changes of zero-sodium artificial seawater.

Natural seawater, adjusted to pH 7 with 1 M HCl, was used to treat unfertilized eggs with *clasto*-lactacystin β-lactone at a final concentration of 100 μM. *Clasto*-lactacystin β-lactone (Calbiochem, La Jolla, CA) was dissolved in dimethyl sulfoxide at 50 mM,

aliquoted, and stored at −20°C. *Clasto*-lactacystin β-lactone is unstable at pH 8, the pH of natural seawater. Eggs were treated with *clasto*-lactacystin β-lactone-containing seawater for 4–7 h, and pH_i was not decreased by this treatment (data not shown).

Microinjection

Microinjection into sand dollar eggs and quantification of injection volumes were done according to the methods of Hiramoto (1974) and Kishimoto (1986).

Optical Equipment and Recording Fluorescence Intensity

To determine the initial rate of hydrolysis of Suc-Phe-Leu-Arg-CAMS in Figs. 1 and 3, we used the same equipment and procedure as described previously (Chiba *et al.*, 1997). The ARGUS image processing system (Hamamatsu Photonics K. K., Japan) was used to measure fluorescence intensity increase from substrate hydrolysis for data in Tables 1, 2, and 3.

To estimate pH_i, an inverted light microscope (DMIRB; Leica) was connected by an adapter tube to a HiSCA CCD camera (C6790) of the ARGUS/HiSCA image processing system (Hamamatsu Photonics K. K.). Excitation light from a xenon lamp was alternated between 450 and 490 nm under computer control (C6789; Hamamatsu Photonics K.K.). The emitted light passed through a dichroic beam splitter at 510 nm and through a 515- to 560-nm emission filter (Leica). The ratios of the emission intensities at 490/450 nm were calculated using the ARGUS/HiSCA image processing system (Hamamatsu Photonics K.K.).

TABLE 1

Changes in V_0 of Suc-Phe-Leu-Arg-CAMS *in Vivo* after Fertilization in Natural Seawater

Time after fertilization	*Clasto*-lactacystin β-lactone	V_0 (average of relative intensity) ± SEM	*N*
Unfertilized	−	1.9 ± 0.86*	16
	+	0.0 ± 1.50*	2
1–2 min	−	16 ± 1.44	4
8–15 min	−	17 ± 1.89**	4
19–49 min	−	12 ± 0.94**	12
58–71 min	−	12 ± 1.43**	6
11–60 min	+	5.7 ± 1.53**	7

Note. The experiments were performed at 22°C. Development progressed as follows: The male and female pronuclei were adjacent by 15 min postfertilization. Centering of the pronuclei was complete by 17 min postfertilization. Pronuclear fusion, centrosome replication, and establishment of the bipolar spindle were complete by 26 min postfertilization. Nuclear envelope breakdown was complete by 50 min postfertilization. Anaphase B (spindle elongation) was complete by 62 min postfertilization, and the first cytokinesis was complete in 50% of the embryos by 66 min postfertilization. *Clasto*-lactacystin β-lactone + indicates that the eggs were treated for 6 h with 100 μM *clasto*-lactacystin β-lactone before fertilization or before substrate microinjection in the case of unfertilized eggs.

* $P < 0.0001$, unpaired *t* test for unfertilized eggs with and without *clasto*-lactacystin β-lactone.

** $P < 0.0003$, unpaired *t* test for fertilized eggs with and without *clasto*-lactacystin β-lactone.

RESULTS

After the fluorogenic substrate Suc-Phe-Leu-Arg-CAMS was microinjected into a sand dollar egg, excitation at 380 nm was used to monitor the release of fluorescent ACMS resulting from peptidase hydrolysis of the amino acid–arylamide bond after arginine. Bestatin was co-injected with the substrate to inhibit aminopeptidase, which also hydrolyzes this substrate (Chiba *et al.*, 1997). Since the rate of hydrolysis was constant for at least a few min, V_0 could be calculated (Fig. 1 and Table 1). These data indicate that protease activity increased rapidly after fertilization, reached a maximal rate by 10 min after fertilization, and remained high throughout the first cell cycle.

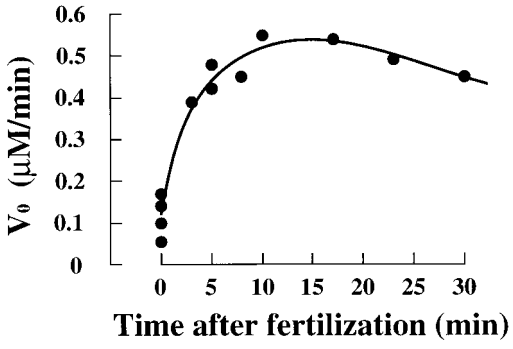

FIG. 1. Changes in the V_0 of the proteasome substrate Suc-Phe-Leu-Arg-CAMS after fertilization. To calibrate the rate of hydrolysis, the known fluorescent product of hydrolysis (ACMS) at a concentration of 6.3 μM was injected into each egg, along with the exopeptidase inhibitor, bestatin. Next, the fluorogenic substrate Suc-Phe-Leu-Arg-CAMS was injected at the indicated time to get values of V_0. Bestatin and the substrate were injected to final concentrations of 800 and 63 μM, respectively.

To demonstrate that this peptidase activity was dependent on the proteasome, we treated sand dollar eggs with a specific inhibitor of proteasome activities, *clasto*-lactacystin β-lactone (Omura *et al.*, 1991a,b; Fenteany *et al.*, 1995; Dick *et al.*, 1996). Table 1 shows that treatment with the inhibitor significantly decreased V_0. This suggests that Suc-Phe-Leu-Arg-CAMS is hydrolyzed by the proteasome. Direct measurement of pH_i in the developing sea urchin egg has demonstrated that fertilization induces a rise in pH_i (Shen and Steinhardt, 1978). Fertilization of the sand dollar egg also results in a sodium-dependent pH_i rise (Fig 2). pH_i in living sand dollar eggs can be measured after microinjection of the pH-sensitive fluorescent dye BCECF. Dye covalently linked to a 10-kDa dextran is not lost from the cell nor localized in noncytoplasmic cellular compart-

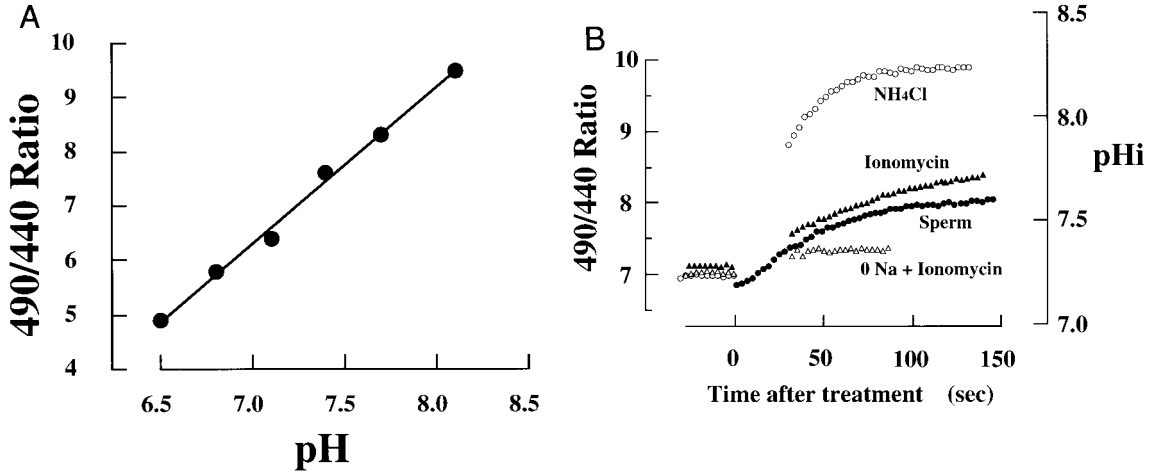

FIG. 2. (A) *In situ* titration of BCECF–dextran. In normal seawater, unfertilized eggs were injected with BCECF–10 kDa dextran to a final concentration of 20 μM. The seawater was replaced with a model intracellular medium containing (in mM): glycine, 300; KCl, 175; mannitol, 185; NaCl, 20; $MgCl_2$, 5; Hepes, 25; Pipes, 25; titrated to the indicated pH with KOH. Then, while fluorescence was recorded, the medium was replaced again with the same model intracellular medium plus 100 μM digitonin to permeabilize the eggs. The ratio of the fluorescence intensities at the two excitation wavelengths (490/440) was plotted versus the pH of the bathing medium. Symbols represent the mean values of two to four eggs at each pH. (B) Representative traces of pH_i after activation. The pH_i of unfertilized eggs was monitored during the change from natural seawater to pH 9 natural seawater containing 15 mM NH_4Cl (open circles, $n = 2$), during activation by fertilization (closed circles, $n = 4$), during activation with 1 μM ionomycin in natural seawater (closed triangles, $n = 2$), and during activation with 1 μM ionomycin in sodium-free artificial seawater (open triangles, $n = 3$). BCECF–dextran fluorescence ratios (left y axis) and pH_i (right y axis) calibrated from (A).

ments. Figure 2A shows *in situ* titration of the BCECF–dextran microinjected into unfertilized sand dollar eggs. Following replacement of the seawater with a model intracellular medium buffered to varied pH, the eggs were permeabilized with digitonin. The ratio of emission intensities from alternate excitation with 490- and 440-nm light increased linearly for pH buffered from 6.5 to 8.1. Using these intracellular calibration data, Fig. 2B shows the change in pH_i for unfertilized sand dollar eggs before and after various treatments. Ionomycin treatment and sperm addition in natural seawater both resulted in an increase in pH_i of about 0.4 pH units. Treatment with 15 mM NH_4Cl in pH 9 natural seawater resulted in a rise in pH_i of about 0.9 pH unit. The intracellular pH of unfertilized, unactivated eggs in zero-sodium artificial sea water was not significantly different from the intracellular pH of unfertilized, unactivated eggs in natural seawater (Fig. 2B, −25 min to 0 min). The addition of 1 μM ionomycin to eggs in zero-sodium sea water resulted in an attenuated rise in intracellular pH of about 0.1 pH unit, most likely the result of a reduction in sodium–hydrogen exchange.

The pH_i of unfertilized sand dollar eggs was artificially elevated using pH 9 natural seawater containing 15 mM ammonium chloride (Fig. 2B). Table 2 shows that V_0 was accelerated in NH_4Cl-treated unfertilized eggs to rates equal to or greater than those measured following fertilization. For unfertilized eggs pretreated with *clasto*-lactacystin β-lactone, substrate hydrolysis measured after exposure to NH_4Cl-containing seawater was reduced about 70%.

Both $[Ca^{2+}]_i$ (Steinhardt *et al.*, 1977; Hamaguchi and Hamaguchi, 1990) and pH_i (Shen and Steinhardt, 1978) increase following fertilization in echinoderm eggs. To increase $[Ca^{2+}]_i$ without elevating pH_i, unfertilized eggs were exposed to the calcium ionophore ionomycin in zero-sodium artificial seawater (zero-Na^+ ASW). The lack of external sodium ion prevents the stimulation of Na^+/H^+ exchange that is triggered by the $[Ca^{2+}]_i$ rise at fertilization

TABLE 2

Treatment	V_0 (average of relative intensity) ± SEM	N
Unfertilized in natural seawater	4.0 ± 1.00	3
15 mM NH_4Cl[a]	32 ± 2.29*	10
Clasto-lactacystin β-lactone + 15 mM NH_4Cl[a]	9.4 ± 1.21*	7

[a] Eggs were pretreated in pH 7 seawater for 7 h with or without 100 μM *clasto*-lactacystin β-lactone. Then the medium was changed to 15 mM NH_4Cl in pH 9 seawater 15 min before substrate microinjection.

* $P < 0.0001$, unpaired t test for NH_4Cl-treated eggs with and without *clasto*-lactacystin β-lactone.

TABLE 3

Treatment	V_0 (average of relative intensity) ± SEM	*N*
Natural seawater	5.4 ± 1.12	5
Ionomycin in natural seawater	24 ± 1.73*	3
Zero-Na^+ artificial seawater	4.0 ± 1.00	3
Ionomycin in zero-Na^+ artificial seawater	3.0 ± 0.50*	9

Note. Unfertilized eggs were treated with or without 1 μM ionomycin in natural seawater or zero-Na^+ artificial seawater.

* $P < 0.0001$, unpaired *t* test for ionomycin-treated eggs in natural seawater or in zero-Na^+ artificial seawater.

or by calcium ionophore exposure (Shen and Steinhardt, 1979). Although the absence of external Na^+ also inhibits hardening of the fertilization envelope (Schuel *et al.*, 1982), treatment with calcium ionophore in zero-Na^+ ASW causes the elevation of $[Ca^{2+}]_i$ and cortical granule exocytosis in sea urchin eggs (Shen and Steinhardt, 1979). We also observed cortical granuole exocytosis and the transient rise of low fertilization envelopes for calcium ionophore-treated sand dollar eggs in zero-Na^+ ASW. Since the calcium ionophore A23187 is highly fluorescent, we used ionomycin, which is not excited by 380-nm light.

In natural seawater, which has about 486 mmol Na^+ per liter, treatment of unfertilized eggs with ionomycin stimulated the rate of hydrolysis of the proteasome substrate, with an increase in activity similar to the levels following fertilization (Table 3). To test if this increased activity was due to $[Ca^{2+}]_i$ elevation or due to the rise in pH_i stimulated by calcium ion, unfertilized eggs were treated with ionomycin in zero-Na^+ ASW. As shown in Table 3, ionomycin treatment in the absence of external Na^+ does not stimulate proteasome substrate hydrolysis.

To confirm that pH_i rise without an increase in $[Ca^{2+}]_i$ is sufficient for proteasome activation, pH buffers were microinjected into unfertilized eggs to stabilize pH_i from 7.1 to 8.0. The buffers contained 100 mM EGTA to minimize $[Ca^{2+}]_i$ rises induced during the injection procedure, and a lack of cortical reaction supported effective buffering of Ca^{2+}. Figure 3 clearly indicates that pH_i change alone is sufficient for the activation of proteasome in unfertilized eggs. These results suggest that the $[Ca^{2+}]_i$ rise that occurs at fertilization does not stimulate proteasome activity, but instead serves as a trigger to activate the Na^+/H^+ exchange which is responsible for a rise in pH_i. Proteasome peptidase activity appears to be directly activated by a rise in pH_i.

On the other hand, injection of pH 7.1 buffer into fertilized eggs did not decrease substrate hydrolysis significantly: V_0 for before and after injection of the buffer were 0.23 ± 0.052 and 0.20 ± 0.033 μM/min, respectively. This result suggests that once the proteasome is activated by the rise in pH_i following fertilization, later modulation of proteolysis rate is largely dependent on factors other than pH and that pH only has to be in a permissive range for a transient period for this step of the activation process.

DISCUSSION

The molecular mechanisms which follow the $[Ca^{2+}]_i$ and pH increase at fertilization and release the quiescent egg from the G1 check point are still unclear. However, regulated proteasome activity is known to be a necessary element in the precise timing of events during the cell cycle (reviewed by King *et al.*, 1996). Progress through the cell cycle depends on the alternate accumulation and destruction of proteins known as cyclins. The interaction of cyclins with activated cyclin-dependent kinases promotes the transition from G1 to S phase, from G2 into prophase, from metaphase into anaphase, and from telophase into G1. Cyclin-dependent kinases are regulated by specific inhibitors and activators. Both the cyclins and the activators and inhibitors of cyclin-dependent kinases are potential substrates for proteasome proteolysis. For example, during early G1 in budding yeast, the kinase inhibitor Sic1 prevents precocious DNA synthesis by inhibiting S-phase cyclin-dependent kinases. However, in late G1, the assembly of G1 cyclin-dependent kinases results in phosphorylation of Sic1, which initiates the ubiquitination of this S-phase cyclin-dependent kinase inhibitor that facilitates its destruction by the proteasome. A series of enzymes in the E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin-protein ligase) families promotes the protein polyubiquitination which leads to

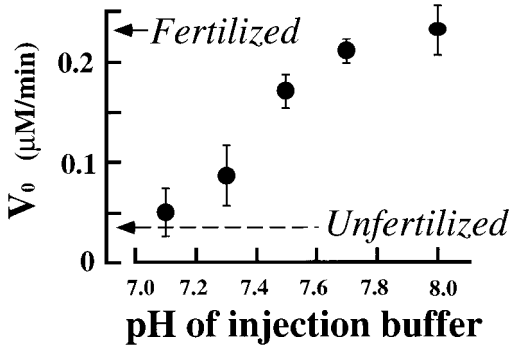

FIG. 3. Changes in the initial velocity of hydrolysis of Suc-Phe-Leu-Arg-CAMS in unfertilized eggs microinjected with buffers at various pH values. Eggs were microinjected with the buffer containing 300 mM Hepes, 300 mM Pipes, and 100 mM EGTA, pH 7.1–8.0. EGTA was included in the buffer to prevent possible injection-induced activation of the eggs. One minute after injection of the buffer, the substrate was injected to determine V_0. Symbols represent the mean values ± SE ($N = 3$ or 4). Dashed lines indicate the mean values of V_0 in fertilized or unfertilized eggs without injection of pH buffer: fertilized, 0.23 ± 0.052 μM/min; unfertilized, 0.033 ± 0.018 μM/min (the mean values ± SE, $N = 3$).

proteolysis in the 26S proteasome. A family of phosphatases selectively removes ubiquitins from polyubiquitinated substrates. Thus a delicate balance of ubiquitin addition and removal determines the rate and specificity of 26S proteasome degradation of different cell cycle proteins.

Although most cyclins are regulated by ubiquitination and degraded by the proteasome, at least one, cyclin D_1, is regulated by calpain (Choi *et al.*, 1997). The cyclosome or anaphase-promoting complex regulates ubiquitin ligase activity (reviewed by Townsley and Ruderman, 1998). This ligase is activated by phosphorylation at the end of M phase. Other ubiquitin ligases are thought to be constitutively active and act on phosphorylated substrates. In each case, cell-cycle-dependent phosphorylation is the key step for the ubiquitination, which is prerequisite for degradation by the proteasome (reviewed by Hershko, 1997). The activity of isolated proteasomes is also reported to be regulated in a manner that is dependent on cell cycle in ascidian embryos (Kawahara and Yokosawa, 1994). The activity of the proteasome has also been postulated to be dependent on the increase in $[Ca^{2+}]_i$ at fertilization. Using *Xenopus* oocytes frozen at various times after activation with calcium ionophore, the activity of isolated proteasomes peaked between 2 and 5 min after ionophore addition (Aizawa *et al.*, 1996). Aizawa and co-workers measured fractionated activity of the 26S proteasome and found that the activity was enhanced transiently and then decreased during the metaphase–anaphase transition triggered by treatment with calcium ionophore A23187. Since the proteasome activation was completely abolished by pretreatment with a cell-permeable calcium-chelating agent, BAPTA-AM, they concluded that the activity was regulated by intracellular calcium mobilization.

Two important regulators of the 20S proteasome have recently been discovered: PA 28 (Ma *et al.*, 1992) affects the peptidase activity of the 20S proteasome while PA 700 (Akaishi *et al.*, 1995) regulates the degradation of intact proteins. When bovine PA 28 was added to purified lobster muscle proteasomes, peptidase activities were stimulated as much as 99-fold and the pH optimum of the acidic chymotrypsin-like activity was shifted from pH 6–6.5 to pH 7–7.5 (Mykles, 1996). Although evidence for regulation of the proteasome by calcium ion has been found *in vitro* (Realini and Rechsteiner, 1995; Nakajima *et al.*, 1998), the high levels required appear not to be physiological. Still, locally high calcium ion "hot spots" may be common in living cells (Tucker and Fettiplace, 1996).

Measurement of proteasome activity in the complex environment of the living cell avoids possible artifacts associated with loss of regulatory elements during proteasome isolation. Measurement of proteasome activity *in vivo* was made possible by the development of a water-soluble fluorogenic amine, ACMS (Sato *et al.*, 1988). After modification to enhance solubility in the cytoplasmic environment, a proteasome substrate that released ACMS upon peptidic hydrolysis was microinjected into starfish oocytes to study proteasome activation during hormone-induced maturation (Chiba *et al.*, 1997). In our current study we microinjected this same substrate into eggs of the Japanese sand dollar, *C. japonicus*, to measure the trypsin-like peptidase activity of the proteasome following activation either by fertilization or by parthenogenetic agents.

Our results show that activation of the proteasome can be attributed to the rise in intracellular pH after fertilization and that an increase in intracellular free calcium alone is not sufficient to stimulate proteasome activity. The initial velocity of hydrolysis was measured after the cell-impermeant fluorogenic substrate was microinjected into an unactivated or activated sand dollar egg. The V_0 in the unfertilized egg was low. Following fertilization, the V_0 of the proteasome substrate had increased 5- to 9-fold by 10 min after fertilization. During this same interval, pH_i, measured using BCECF–dextran, rose from 7.2 to 7.6. A similar rise in pH_i after fertilization has been well documented in sea urchin eggs (Johnson *et al.*, 1976; Shen and Steinhardt, 1978, 1979). The rise of pH_i after fertilization occurs via Na^+/H^+ exchange, which is activated by a transient $[Ca^{2+}]_i$ rise at fertilization (Whitaker and Steinhardt, 1985). Elevated pH_i is maintained during early development (Shen and Steinhardt, 1978, 1979).

Since both pH_i and $[Ca^{2+}]_i$ increase following fertilization, the role of these two ions in proteasome activation was unclear. To separate these variables, we used artificial treatments to elevate pH_i and $[Ca^{2+}]_i$ in unfertilized eggs. The calcium ionophore, ionomycin, in natural seawater causes $[Ca^{2+}]_i$ elevation followed by pH_i elevation (Shen and Steinhardt, 1979). Ionomycin applied in zero-sodium artificial seawater results in increased $[Ca^{2+}]_i$ without increase in pH_i because Na^+/H^+ exchange is required for the rise in pH_i (Johnson *et al.*, 1976; Shen and Steinhardt, 1978, 1979). Zero-sodium artificial seawater could not be used for activation by sperm as Na^+ is required for fertilization. As previously observed for sea urchin eggs, ionomycin treatment of unfertilized sand dollar eggs in natural seawater resulted in cortical granule exocytosis, elevation of the fertilization envelope, and a transient increase in Calcium Green fluorescence intensity, all indicating a rise in $[Ca^{2+}]_i$ (reviewed by Whitaker and Steinhardt, 1982, 1985). When we measured proteasome activity and pH_i in unfertilized eggs exposed to calcium ionophore in sodium-free seawater, neither pH_i nor V_0 of the proteasome substrate was increased. Cortical granuole exocytosis, elevation of a fertilization envelope, which failed to harden due to low external Na^+ (Schuel *et al.*, 1982), and a transient increase in Calcium Green fluorescence intensity all indicated that a rise in $[Ca^{2+}]_i$ had occurred in response to ionomycin in the zero-sodium ASW.

Unlike Rees *et al.* (1995), we did not observe an acidification on placing unfertilized eggs into zero-sodium ASW. We suspect that their anomalous results with pH and initiation of protein synthesis are the result of the condition of animals whose gametes were retained for long periods under suboptimal conditions or may have been handled roughly during solution changes. We suspect this for three

reasons. Foremost, their eggs appear to have high pH values in the unfertilized state, 7.3 instead of the 6.9 of previous studies of *L. pictus* by both microelectrode and BCECF–dextran methods (Shen and Steinhardt, 1978; Shen and Buck, 1990). Second, measurements of protein synthesis were not as sensitive to inhibitors of the sodium/proton exchangers as previously seen. This is not surprising given that their unfertilized eggs were already at the pH that is permissive for protein synthesis. Finally, the intracellular pH of unfertilized eggs dropped in zero-sodium ASW in their experiments. The simplest explanation is that somehow the unfertilized eggs in Rees *et al.* (1995) had a partially active sodium/proton exchanger and were sodium-loaded. Placing them in zero-sodium ASW would therefore allow the sodium/proton exchanger to run backward and acidify the cytoplasm.

In additional experiments, microinjection of buffers to elevate pH_i without $[Ca^{2+}]_i$ change resulted in activation of the proteasome. Thus, pH_i rise after fertilization was sufficient to activate the proteasome in the sand dollar egg. Proteolytic enzymes are known to be synthesized as inactive precursors called zymogens (reviewed by Khan and James, 1998). Autocatalytic processing has been found to be required for activity of the 20S proteasome of *Thermoplasma acidophilum* (Seemüller *et al.*, 1996). The intracellular pH rise after fertilization may trigger proteasome activation by stimulating association with regulatory subunits, such as PA 28 or PA 700 (Hendil *et al.*, 1998), autocatalysis, or other enzymatic alteration.

As summarized above, in budding yeast, the G1- to S-phase transition is induced by the proteasome-dependent degradation of Sic1, an inhibitor of cyclin-dependent kinase (Cdk) (Schneider *et al.*, 1996). Also, in quiescent mammalian cells, a Cdk inhibitor, $p27^{kip1}$, is rapidly degraded by the ubiquitin/proteasome system when cells are stimulated to grow (Hershko, 1997). If such inhibitors exist in unfertilized echinoderm eggs, proteasome activation by pH_i rise might be a key step for degradation of inhibitors and exit from G1 at fertilization. These results suggest that future work might be usefully done to see if proteasome activation by pH is a crucial part of the release from G1 at fertilization. Previous work on synchronous nuclear division in *Physarum* has shown that intracellular pH must be in a permissive range at least for a transient period during the preparation for mitosis (Morisawa and Steinhardt, 1982).

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